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NAME OF CANDIDATE:	**Shriya Rajendra Sawant**
DOCTORAL COMMITTEE:	**(i) Dr. Kayzad Nilgiriwala,** Research Officer, Foundation of Medical Research, Mumbai **(ii) Dr. Binoj Kutty,** Assistant Professor, St. Xavier's College, Mumbai
NAME AND DESIGNATION OF MENTOR:	**Dr. Harinder Singh** Assistant Professor, Sunandan Divatia School of Science, SVKM's NMIMS University, Vile Parle (W), Mumbai – 400 056
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PLACE OF RESEARCH:	Sunandan Divatia School of Science, SVKM's NMIMS University, Vile Parle (W), Mumbai- 400 056
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SIGNATURE OF THE CANDIDATE:	Sawant
SIGNATURE OF THE MENTOR:	Harindersingh

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Chapter 1: Introduction

In India, oral cancer (OC) is the second most frequent malignancy, and survival rates have not improved in recent decades. Oral cancer is the most prevalent cancer in men in India, with 104,661 cases reported. It is also the fourth most common cancer in women, with 31,268 cases reported, accounting for nearly 36% of the worldwide burden of oral cancer (Sung et al., 2021). The buccal mucosa is the most afflicted area, followed by the lateral surface of the tongue, base of the tongue, lips, and hard palate. Oral squamous cell carcinoma (OSCC) is the term used to describe a cancer that arises from the mucosal lining of the mouth which contributes to about 90% OC cases (Warnakulasuriya, 2018).

The high prevalence of OC in India is related to tobacco/areca nut chewing, alcohol intake, and Human Papillomavirus types HR-HPV16/18, with tobacco use beginning at the age of 9-12 years on average (Coelho, 2012). Tobacco is extensively used in Asian countries, both in smoking and non-smoking forms. Cigarettes, bidis, and hookah are all forms of smoking, with bidi smoking having the highest risk of OC when compared to cigarettes and hookah. In India, smokeless tobacco has been introduced in a variety of forms, including pan masala and gutka, which are tobacco, areca nut, lime, and catechu mixes that are highly carcinogenic and genotoxic (Chaturvedi et al. 2019). Alcohol usage, which includes the frequency of consumption, the kind of alcohol consumed, and the consumer's geographic location, all contribute to the risk of oral cancer. Tobacco chewing and smoking have a synergistic impact with alcohol towards progression to OC (Ghantous et al. 2018).

Another risk factor for OC is diet, which is influenced by lifestyle, socioeconomic level, and culture. OC is also connected to poor oral hygiene, leading to different infections and periodontal illnesses, which involves role of bacterial in causing inflammation and eventually instigate carcinogenesis (Rodríguez-Molinero et al. 2021). Recent findings in cancer research suggest a link between the presence of specific types of bacteria and the development of cancer in the human body, the reports are based on genome sequencing. Dysbiosis, or microbial imbalance, is characterised by a decrease of microbial diversity, the loss of helpful bacteria, and the increase of pathogenic germs. These factors can contribute to the development of cancer (Kakabadze et al. 2020).

In the current work, the oral microbiota of the Indian OC population and tobacco chewers was identified and compared to healthy individuals. From the microbiome data, we also identified the microbial biomarkers for three studied groups. A simple qPCR-based method was developed that would aid in early diagnosis of OC in high-risk individuals. A preliminary investigation was performed to understand the host-pathogen interactions at the cellular and molecular level. The resulting data would help to distinguish the oral microbiota of Indians from that of the rest of the globe, as well as add to the global human oral microbiota knowledge pool. It will also improve our understanding of the complexities of bacterial pathogenesis in oral cancer.

Chapter 2: Review of Literature

2.1 Bacteria in Oral Cancer

The oral microbiota is composed of around 600 different bacterial species. The majority among these species, however, are uncultivable. The advent of modern sequencing technologies allows the discovery of bacterial populations that live in the mouth and are involved in human health (Dewhirst et al. 2010; Proctor et al. 2019). The most prevalent schemas proposed to describe the pathophysiology involving microbiota in the development of cancer (la Rosa et al. 2020) are:

i) Chronic inflammation and immune responses that can promote carcinogenesis and tumour growth
ii) Metabolic activity changes that lead to increased production of toxic and carcinogenic metabolites
iii) Alteration of epithelial barrier integrity and epigenetic modulations induced by microbiota.

Periodontal disorders and chronic inflammation are linked to anaerobic oral bacteria such as *Fusobacterium, Porphyromonas*, and *Prevotella* species. Inflammatory mediators released by bacteria have the ability to interact with cells in several tissues, causing dispersed inflammation. Expression of Interleukins (IL-1, IL-6, IL-17, IL-23), Tumour Necrosis Factor (TNF-), and Matrix Metallo-Proteinases (MMP-8, -9 and -13) are all influenced by periodontal bacteria (Szkaradkiewicz and Karpiński 2013; Lamont et al. 2018). Sulphur compounds, acids, and free radicals, primarily nitric and oxygen reactive species, are produced by bacterial metabolism and can cause genetic damage. Moreover, certain bacteria have an alcoholic metabolism that produces acetaldehyde, which is necessary for neoplastic transformation (Karpiński 2019).

2.2 Oral Cancer Microbiome Studies

One of the earliest reports published on the oral microbiome and OC was by Nagy et al., and they analysed the biofilm present on the surface of OSCC tissue and healthy contagious mucosa. The study showed increased numbers of anaerobes such as *Veillonella,*

Fusobacterium, Prevotella, Porphyromonas, Actinomyces and *Clostridium*, and aerobes such as *Haemophilus, Serratia liquefaciens, Klebsiella pneumoniae Citrobacter freundii, Enterobacteriaceae* and *Streptococcus β-haemolyticus* (Nagy et al. 1998). In another pilot study reported by Pushalkar et al., microbiome analyses were performed using 16S rRNA sequencing of saliva samples collected from three OSCC cases and Denaturing Gradient Gel Electrophoresis (DGGE) was used to compare the profile with two matched controls. OSCC samples predominantly contained organisms from the genera *Streptococcus, Gemella, Rothia, Peptostreptococcus, Porphyromonas*, and *Lactobacillus* (Pushalkar et al. 2011). Later the researchers reported the oral microbiota from ten OSCC tumor samples using the same sequencing technique (Pushalkar et al. 2012). They found phylae Firmicutes, Class *Bacilli* consisting order *Lactobacillales* (54.8%) and *Bacillales* (11.8%) to be predominant in the tumor tissues. The tumor library was predominated by *Streptococcus* (50.8%), *Gemella* (11.6%), *Parvimonas* (4.6%), *Peptostreptococcus* (2.8%), *Xanthomona*s (2.4%), *Johnsonella* (1.6%), *Solobacterium* (1.6%), *Atopobium* (1.2%). *Eubacterium [11] [G-3], Campylobacter* and *Catonella* were exclusive to tumor library, which was a new panel of organisms to be associated with OSCC from the yet discussed studies, and required further validations. This may also suggest that the change in oral health of an individual can cause dysbiosis in the oral cavity that can turn a commensal bacterial population to pathogenic.

Another microbiological study, performed by Cankovic et al. (2013), was aimed to observe the difference in microflora of OSCC swab samples when compared to oral mucosa lesions. The microbiological assays depicted increased pathological flora and decreased normal flora in samples of the OSCC group, when compared to the control group, a common observation found in most of the studies reported (Čanković et al. 2013). Schmidt and colleagues studied the bacterial abundance in OC samples (Schmidt et al. 2014). In both the studies, presence of *Firmicutes* and *Actinobacteria* decreased in the OC samples as compared to healthy counterparts. Further, pre-cancerous samples also showed a similar decrease. Such studies suggest alterations in microbiota may occur very early on in cancer development during pre-cancer stages. At genus level, significant reduction in the abundance of *Streptococcus* and *Rothia* and a corresponding increase in *Fusobacterium* was reported in OSCC samples compared to their matched controls. Thus, such organisms can be used as microbial biomarkers to study the progression of cancers.

Bolz et al. performed a microbiological study to isolate oral bacteria from oral cancer, high-risk individuals and control patients. The ratio of aerobes to anaerobes was found to be 1:1.6 in control samples, equal ratio (1:1) in high-risk patients whereas the tumor patients displayed a reversal of ratio (0.5:1). This depicts an increase in abundance of anaerobic bacteria in tumor samples (Bolz et al. 2014). This could be due to dysbiosis and physiological changes during tumorigenesis, which favours the dominance of anaerobes. A similar microbiological study reported an increased median CFU/ml in saliva and swab of the oral cavity obtained from OSCC patients as compared to healthy controls. Increased abundance of *Candida* spp, particularly *Candida albicans* was isolated from the carcinoma site (Metgud et al. 2014). OC samples had more bacterial species than healthy persons, according to Zhao et al. The most overexpressed bacteria in OSCC samples with past periodontitis are *Catonella, Dialister, Filifactor, Fusobacterium, Parvimonas, Peptococcus,* and *Peptostreptococcus*. The examination of *Fusobacterium* bacteria, in particular, has been considered as a diagnostic criterion for OSCC (Zhao et al. 2017). The number of Fusobacteria was considerably greater in OC patients who were progressing, especially in stage-IV patients (7.92%) compared to healthy controls (2.98%) (Yang et al. 2018). Lim et al (181) analysed oral rinse to evaluate the microbiome variations and the results indicated *Capnocytophaga gingivalis*, *Peptostreptococcus sp., Porphyromonas gingivalis, Prevotella sp*., and *Streptococcus* sp. as the oral microorganism mostly associated with OSCCs (Lim et al. 2018).

2.3 Effect of Tobacco on Oral Microflora

It is a well-established fact that use of tobacco (smoking or chewing) makes an individual more susceptible to chronic health conditions including pulmonary diseases, various cancers, periodontal diseases, stroke etc. However, use of tobacco also makes an individual more prone to bacterial infections. This occurs due to effect of tobacco, making the bacterial population in the organ affected relatively unstable and diverse as compared to healthy non-tobacco using individuals (Bagaitkar et al. 2008). Comparison of the oral biofilms between tobacco users and non-users showed increased pathogenic bacterial species such as *Fusobacterium, Cardiobacterium, Synergistes, Selenomonas, Haemophilus* and *Pseudomonas* in the tobacco users. This depicts the use of tobacco favours early acquisition and colonization of pathogens in oral biofilms (Kumar et al. 2011).

There are three mechanisms by which tobacco can increase risk of infection by pathogenic/opportunistic bacteria:

i) Tobacco induced physiological and structural changes in humans
ii) Tobacco induced increase in bacterial virulence
iii) Tobacco induced deregulation of immune function

Hence, the microbiota alteration due to habits such as tobacco chewing can also lead to progression towards carcinogenesis. The microbial diversity of the oral cavity in Indian population of long-term tobacco chewers and OC patients is unexplored. Microbiome research is still in its early stages. A lot of research is being done, and data is being uploaded on a regular basis. Studies with a bigger sample size and several sites in health and illness are required to be discovered to have consistent trends and provide actual results. This will help to find distinct biomarkers and aid in targeted medicines and customised medicine in clinical practise for improved patient management.

Chapter 3: Lacunae, Rationale, Aim and Objectives

3.1 Lacunae/Rationale

Oral cancer (OC) the most common cancer observed in Indian males. Risk factors of OC include major lifestyle factors such as tobacco chewing, alcohol consumption, smoking which are widely observed in the Indian and South Asian subcontinent. Apart from these, there has been a rise in the OC cases in individuals who do not follow the said lifestyle habits. This led to the curiosity for identification of newer risk factors associated with OC, where microbiota was found to be a potential risk factor. Several studies have demonstrated the necessity of a normal, healthy microbiota in supporting good health, and dysbiosis can lead to issues such as cancer. The human body's healthy and altered microbiota, including the oral microbiome and its role in disorders including periodontitis, dental caries, and oral cancer; is being studied extensively across the world, however there is a lack of understanding about the oral microbiome in the Indian population.

The Indian population offers a wide scope of individuals following wide variety of lifestyle factors including tobacco chewing, smoking, alcohol consumption, etc. Thereby the Indian population acts as a suitable demography to study the OC microbiome due to the high incidence rate as well as exposure to the risk factors. This would aid us in identifying the oral microbiome of Indian OC patients, tobacco chewing population and compare with the healthy individuals. The study would also help us identify microbial biomarkers, if any found in the Indian population for the study groups. This data can eventually help in identification of risk factor for diagnosis as well as treatment strategies. The biomarkers identified in the OC group can help in early diagnosis of individuals at high risk of developing OC, such as tobacco chewers/alcohol consumers/smokers. Along with identification, it is essential to unveil the mechanism of action of significant bacteria which causes gene dysregulation and thereby progression to oral cancer.

3.2 Aim and Objectives

3.2.1 Aim

To identify bacterial diversity in oral cavity and its association with Oral Cancer.

3.2.2 Objectives

1. To identify the bacterial diversity in oral cavity of oral cancer patients and tobacco chewers.
2. To find the correlation of oral bacterial diversity and oral cancer.
3. To study host-microbe interaction at molecular level.

Chapter 4: Identification of Bacteria in Oral Cavity of Oral Cancer Patients and Tobacco Chewers in Indian Population

This chapter deals with the 16S rRNA metagenomics study performed for identification of bacterial diversity in oral cavity in three study groups namely, healthy controls, long term tobacco chewers and oral cancer patients. Following sample collection from participants, DNA was isolated using silica column-based method for efficient isolation and 16S rRNA targeted Next Generation Sequencing was performed. The raw data obtained was subjected to analysis using QIIME2™ pipeline for identification and assessment of bacterial taxonomy.

4.1 Study subjects

The study subjects in the study were divided into three study groups consisting of 40 individuals in each group, namely histopathologically confirmed as patients suffering from Oral Cancer (OC), long term tobacco chewers (T) and healthy control population (C). Informed consent was obtained from the participants after explaining them about the study. Data on participant's medical history, age, gender, occupation, habits of tobacco chewing, alcohol intake, and general oral hygiene questions were recorded. Ethical approvals for the recruitment of healthy, long-term tobacco chewing volunteers and OC patients were obtained from SVKM's Institutional Ethics Committee (NMIMS/IEC/008/2016) and The Ethics Committee of K. J. Somaiya Medical College and Hospital, Mumbai.

4.2 Sample collection and DNA isolation

Patients rinsed their mouths with 20 ml of sterile saline for 30 s and collected into a 50 ml sterile tube (Al-Hebshi et al. 2019; la Rosa et al. 2020). Salivary samples from patients were taken at the pre-operative stage while the patient was NBM (Nil by Mouth). Subjects from healthy and tobacco groups were asked to refrain from eating, drinking, or oral hygiene procedures for at least 1 hr before sample collection. Salivary samples were collected in well-labelled sterile falcon tubes and stored at 4 °C and processed within 48 hrs. The DNA was isolated using Invitrogen DNA Isolation Kit and quantified using BioTek EPOCH2 microplate reader. The quality of the isolated DNA was assessed by performing agarose gel electrophoresis using 1% agarose.

4.3 Next Generation Sequencing using Illumina Miseq

The NGS was performed at Seoul Women's University, South Korea with the collaborative help of Dr. Sathiyaraj Srinivasan. The PCR amplification of bacterial 16S rRNA hypervariable region V6-V8 was carried out using primers B969F (ACGCGH NRA ACC TTA CC) and BA1406R (ACG GGC RGTGWG TRC AA). The whole sequencing process was done using Illumina (Illumina, San Diego, CA). MiSeq libraries were quantified and then subjected to 300-nucleotide paired-end multiplex sequencing(Lalande et al. 2014; Guo et al. 2016).

A total of 6,296,186 forward and reverse sequences of 300-bp were obtained with a mean of 56,219 and median of 51,278 sequences per sample.

4.4 Taxonomic assessment using QIIME2™

Demultiplexed raw reads (V6-V8) from Illumina sequencer were obtained and imported into Quantitative Insights into Microbial Ecology 2 (QIIME™2) pipeline for further analysis (Estaki et al. 2020). The quality of the reads from the sequencer were assessed using FASTQC followed by filtering out low quality reads by denoising using Deblur and assignment into Operational Taxonomic Units (OTUs). Taxonomy was assigned using the GreenGenes database at different taxonomic levels and compared between the study groups. The differential abundance of bacterial genera was analysed using LEfSe (https:// huttenhower. sph. harvard. edu/ galaxy/)(Wang et al. 2019). The Lda Effect Size was analysed using Kruskal Wallis test (threshold=0.05) and Pairwise Wilcoxon test (threshold=0.05).

The reads were classified into 6,733 OTU's using Greengenes database which resulted in identification of 9 phyla, 17 classes, 30 orders, 55 families, 94 genera, and 148 species. The top five phyla observed were Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, and Actinobacteria. The ten most abundant genera observed in all groups were *Streptococcus, Prevotella, Neisseria, Rothia, Haemophilus, Porphyromonas, Paraprevotella, Veillonella, Fusobacterium* and *Leptotrichia.* Of these, *Prevotella* was the most dominant genus in all 3 study groups followed by *Neisseria* and *Streptococcus*. The abundance of *Streptococcus, Neisseria, Rothia* and *Veillonella* was higher in control population as compared to OC patients, whereas abundance of *Prevotella, Porphyromonas* and *Fusobacterium* was higher in OC patients as compared to healthy individuals. 11 genera for tobacco chewing population, 7

genera for OC and nine genera for healthy individuals were identified as microbial biomarkers after performing LEfSe analysis.

4.5 Alpha diversity analysis

Bacterial richness and diversity were analysed by alpha and beta diversity matrices and indices such as Observed OTUs, Shannon index, Simpson index, Chao index, ACE index and Rarefaction curves. The results have been expressed as mean ± SD. Statistical analysis for differences in mean values was performed using one way Analysis of Variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism software (version 8.0.2). p-value < 0.05 was considered statistically significant.

The alpha rarefaction curves depict adequate sampling to perform further analysis. Alpha diversity indices portray highest alpha diversity in tobacco group followed by control group and lowest in OC group. Statistically significant differences were observed between control and OC and tobacco and OC group by alpha diversity indices.

4.6 Beta diversity analysis

To analyse the beta diversity, PERMANOVA, ANOSIM and PERMDISP was performed using QIIME2™ pipeline. Bray Curtis matrix, Jaccard matrix, Weighted and Unweighted Unifrac distance matrices were plotted to assess the distribution of sample compositions in 3D space.

PERMANOVA and ANOSIM demonstrate significant beta diversity between all three study groups suggesting difference in the centroids of the groups. Further, PERMDISP suggests significant difference between control-OC and tobacco-OC groups taking into account the dispersion between the groups. PCA plots show clustering of control and tobacco samples whereas OC samples stretch out away from the C-T cluster. These analyses confirm the bacterial composition of control population is similar to tobacco chewers, whereas that of OC patients is varied from control and tobacco chewers.

4.7 Functional prediction of metabolic pathways

The functional MetaCyc pathways were predicted using PICRUSt2. The results have been expressed as a post-hoc plot using STAMP software. Statistical analysis for significance was

performed using students t-test between groups was followed by Bonferroni correction (Zhang et al. 2020). p value < 0.05 was considered statistically significant.

Pathways related to amino acid synthesis, glucose and sugar utilization were upregulated in control samples as compared to OC group. On the contrary, pathways related to lipid, fatty acid and coenzyme A synthesis were upregulated in OC group. Reductive TCA cycle and pyrimidine biosynthesis pathway were upregulated in tobacco chewers.

Chapter 5: Absolute Quantitation of Oral Bacteria Involved in Oral Cancer and Tobacco Chewers by Real-Time PCR

In this chapter we have exploited quantitative PCR which is an adaptation of PCR, widely used for quantitation of gene expression at mRNA levels as well as for microbial ecology purposes to determine the microbial load and diversity in any environmental sample (Abbott et al. 1988; Nadkarni et al. 2002). Microbial qPCR methods are intended to target highly conserved 16S rRNA genes to measure overall bacterial load in a sample, whereas hypervariable regions of this gene can be targeted for selective amplification and quantification of a specific bacterial population (Smith and Osborn 2009). Because every bacterial species contains varied copies (1–15 copies) of 16S rRNA, this technique has a flaw that must be noted, *i.e.* 16S rRNA gene copies cannot be correctly associated with cell counts (Klappenbach et al. 2000). A housekeeping gene such as RNA Polymerase-subunit gene *(rpoB)*, can be used instead of the 16S rRNA gene. Therefore, we aimed to develop a reliable method for absolute quantification of bacteria using the *rpoB* gene(Case et al. 2007). Assessing the dynamics of the microbial population in diseased conditions can help in determining the mechanisms, as well as abundance of bacteria, which can trigger or develop the cancer.

5.1 Selection of candidate organism and primer designing

Porphyromonas gingivalis, Fusobacterium nucleatum, Capnocytophaga gingivalis, Haemophilus parainfluenzae, Prevotella melaninogenica, Rothia mucilaginosa, Veillonella parvula, and *Streptococcus mutans* were chosen, based on previous reports on oral cavity bacterial species linked to dysbiosis in OC and *rpoB* gene specific primers were used for absolute quantitation(Gholizadeh et al. 2016; Yang et al. 2018). *rpoB* primers were designed against *E. coli* MG1655 as well which was chosen as model organism.

5.2 Establishing standard curve for *E. coli* MG1655

Relation between Ct and CFU/ml was established using *E. coli* as model organism. In brief, 0.1OD culture of *E. coli* was used to obtain CFU/ml using spread plate technique. Parallelly, the culture was used for DNA isolation followed by quantitation and quality assessment of the DNA isolated. The isolated DNA was then serially diluted and subjected to qPCR using

SYBR green chemistry to obtain Ct values, which were used to plot standard curve and equation obtained was used for all further calculations.

The correlation between OD, CFU/mL and DNA concentration (ng/µL) was established where 0.1OD @ 600 nm corresponded to 3.856 (±1.5) X 107 CFU/mL and the DNA isolated from this culture was 3.560 ng/µL. DNA concentration of 0.1OD culture was noted and 6-10-fold dilutions were used to construct the standard curves for *rpoB* gene amplification. The final equation of standard graph obtained was used for all further calculations where, Y is the Ct value for a sample and X is the log of DNA concentration. The abundance is calculated in terms of BGE/ml (Bacterial genomic equivalent/ml)(Sundin et al. 2017).

5.3 PCR analysis for significant bacteria in Oral Cancer

For absolute quantification, primers designed for *rpoB* gene of *P. gingivalis, F. nucleatum, C. gingivalis, H. parainfluenzae, P. melaninogenica, R. mucilaginosa, V. parvula* and *S. mutans* were used. For the PCR, reactions were set up for a total volume of 10 µL followed by melt curve analysis to confirm that only the specific products were amplified. The amplified products were checked on 2% agarose gel for the size of amplified products. All the studies were carried out thrice in triplicates; the results have been expressed as mean ± standard deviation (SD). Statistical analysis for differences in mean values was performed using one way Analysis of Variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism software (version 8.0.2). p value < 0.05 was considered statistically significant.

Significant increase in abundance of *P. gingivalis, F. nucleatum, C. gingivalis, P. melaninogenica* and *V. parvula* in OC patients as compared to healthy individuals. Whereas *H. parainfluenzae, R. mucilaginosa* and *S. mutans* showed higher abundance in healthy controls as compared to OC patients. Variable factors such as age, gender, food, site of cancer, stages of cancer were also assessed.

Chapter 6: Assessment of Host mRNA Expression during Host-Microbe Interaction

This chapter discusses preliminary study performed to assess the host mRNA expression on infection with *S. mutans*, which is a known resident of the oral cavity. Previous reports have demonstrated the role of chronic inflammation and immunological responses in promoting carcinogenesis and tumour growth due to pathogenic bacteria such as *P. gingivalis* and *F. nucleatum* in the development of oral cancer. These studies have not been conducted on the many bacterial species found in the oral cavity, which are essential for optimal oral health. *S. mutans* is an essential organism in maintaining healthy oral cavity, but hardly explored for its effect on expression of genes linked with cancer development.

6.1 Oral Cancer Cell Line procurement and maintenance

The oral cancer cell line (AW13516) used in this study was procured from Tata Memorial Centre- Advanced Centre for Treatment, Research and Education (ACTREC), Navi Mumbai. The cell line was maintained in DMEM containing antibiotics supplemented with 10% FBS. For cryopreservation purposes, the freezing mixture was prepared with FBS: DMSO (9:1).

6.2 Growth and maintenance of *S. mutans*

S. mutans was cultured and maintained in St. Brain Heart Infusion (BHI broth). The culture was freshly grown at 37ºC under anaerobic conditions using anaerobic jar for 48hrs. The culture density was standardized at 0.35OD @ 600nm resulting in 10^8 cells/ml. This culture density and 1:10 serial dilutions are used for infection purposes**.**

6.3 Cytotoxicity assessment for standardization of MOI and time of infection

The extent of cytotoxicity due to bacterial infection was evaluated by MTT assay. After 24hrs of cell seeding, the host cells were infected with *S. mutans* at variable MOI (1,10,100,1000) for variable time periods (0hr.1hr, 3hr, 5hr, 24hr). After infection, at specified time points, 20ul 5mg/ml MTT was added to all the wells and incubated for 2hrs at 37ºC. After incubation, the media from all wells was removed completely and 100µl DMSO was added and the optical density on ELISA microplate reader at 570nm and 650nm (Aggarwal et al. 2019).

The results of cytotoxicity assay showed ~50% cell death at 24hrs infection at all MOIs, but no significant difference was observed at other MOI and times of infection, suggesting the bacterial interaction doesn't hamper host cell viability up to 5hrs of infection.

6.4 Microscopic visualization of infection

Sterile coverslips were placed in each well of 6-well plate for culturing. Host cells were infected with *S. mutans* at MOI of 100 followed by incubation for 1hr, 3hr, 5hr,7hr and 24hrs at 37°C. After the set time of infection was complete, the coverslips were washed in PBS followed by 10% formaldehyde treatment and staining with methylene blue(Brijesh et al. 2006).

The microscopic observation revealed no interaction between host cells and *S. mutans* at 1hr after infection. At 3hrs, the bacteria adhered to the host cell, at 5hrs and 7hrs, the bacteria started to form microcolonies and their density increased. At 24hrs, the host cell death was observed attributed to infection of increased population of *S. mutans,* suggesting the expected interaction between host cells and bacteria must happen between 1hr to 7hrs of infection.

6.5 Evaluation of mRNA expression post infection with *S. mutans*

We next examined the level of mRNA expression in oral cell line after infection with *S. mutans*. We aimed to target at least one gene belonging to pathways of the six-hall marks of cancer. GAPDH was selected as the housekeeping gene for expression analysis. All the studies were carried out thrice in triplicates; the results have been expressed as mean ± standard deviation (SD). Statistical analysis for differences in mean values was performed using one way Analysis of Variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism software (version 8.0.2). p value < 0.05 was considered statistically significant.

mRNA upregulation was observed for Stathmin, CyclinD1, BCL2 whereas downregulation was observed in FGF5 with increased infection duration. IL1b, MMP9, Bax and hIL6 did not show any difference in their expression as the time of infection progressed. This suggests an activation of pathway associated with carcinogenesis.

Chapter 7: Discussion

Microbiome is defined as the collection of microbes present in a given sample, location or ecosystem. The microbiome studies have seen huge progress in the last decade. A famous example is the Human Microbiome Project (HMP), initiated by the National Institutes of Health (NIH) to identify the complete healthy human microbiome in order to appreciate the diversity and complexity of the microbial communities. Similar research initiatives are taken by various countries across the globe like Australia, Canada, France, Germany, Japan, Korea, and India (Dubilier et al. 2015). With the help of newer next generation sequencing technologies, the role and composition of microbiota has been identified in individuals in health and disease, for example cancer.

The oral microbiota in healthy individuals and OC patients has been deciphered in many countries. A few genera predominate the healthy oral microbiota such as *Streptococcus, Gemella, Parvimonas, Peptostreptococcus, Neisseria, Rothia*, *Veillonella*, *Haemophilus, Treponema, Lactobacterium, Eikenella, Leptotrichia, Capnocytophaga, Selenomonas, Granulicatella, Prevotella.* These microbes are normal commensal and generally beneficial, however, some of them can become pathogenic and can aid in progression of healthy cells towards OC under compromised conditions (Aas et al. 2005; Bik et al. 2010; Pushalkar et al. 2012).

In present study, we unearthed the composition of oral microbiota in the healthy Indian population and compared it with the composition of OC patients and long-term tobacco chewers. Indian population belongs to varied demographics and therefore can act as an ideal study population. OC is the most common cancer in Indian males and therefore study of the role of microbiota in Indian OC patients is crucial. Along with this, the use of tobacco in various forms in the Indian population is known to contribute towards high incidence of oral cancer. Therefore, exploring the potential of oral microbiota in tobacco chewing population and their role in OC is of prime importance.

Similar to various reports, we identified Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria as the most common phyla present in the oral cavity of all individuals (Sampaio-Maia et al. 2016; Al-hebshi et al. 2017). The abundance of gram-negative

phyla *i.e.,* Bacteroidetes and Proteobacteria were enriched in OC and tobacco chewing microbiota as compared to gram positive enriched phyla *i.e.,* Firmicutes and Actinobacteria in healthy individuals. The increased abundance of gram negative bacteria in OC has been reported earlier (Bolz et al. 2014). At genus level*, Streptococcus, Prevotella, Neisseria, Rothia, Haemophilus, Porphyromonas, Veillonella, Fusobacterium, Leptotrichia* and *Granulicatella* were the top 10 genera observed contributing to approx. 80% of total bacterial composition. Of these, the abundance of *Streptococcus, Neisseria, Rothia, Veillonella* and *Leptotrichia* was higher in healthy controls. Being commensals, these organisms are known to dominate a healthy oral cavity and maintain healthy functioning (Zaura et al. 2009). On the other hand, increased proportion of bacteria belonging to genera *Prevotella, Haemophilus, Fusobacterium and Porphyromonas* in tobacco chewers and OC patients can be attributed to dysbiosis due to unhealthy oral cavity leading to increased pathogenic bacteria thereby contributing towards the progression towards cancer. The mechanisms of pathogen such as *F. nucleatum* and *P. gingivalis* in OC progression is well known and therefore their increased abundance in unhealthy oral cavity is palpable. *P. gingivalis* is known to increase invasiveness of OC cells by upregulating MMPs and IL8 as well as initiates mesenchymal transition in gingival epithelial cells through ZEB1 (Ha et al. 2016; Sztukowska et al. 2016). On the other hand*, F. nucleatum* uses its adhesin FadA as well as upregulates IL-8 expressions in oral epithelial cells (Gholizadeh et al. 2017).

Although these organisms show significant differences in their abundance in all three study groups, not all could be called as biomarkers when we analyse the entire bacterial composition. Using LEfSe analysis, which along with statistical significance takes into account biological consistency, microbial biomarkers for the study groups were identified. In the tobacco chewing study group, *Leptotrichia, Treponema, Tannerella, Filifactor, Selenomonas* and *Campylobacter* were identified as biomarkers, which are known organisms abundantly known to be present in microbiota of tobacco chewers or OC (Wu et al. 2016; Amer et al. 2017). *Tannerella* and *Treponema* are a part of the red complex which also leads to initiation of periodontitis (Bodet et al. 2006). *Cardiobacterium* was newly identified biomarker for tobacco chewers in our study population.

Similarly, for OC population, *Capnocytophaga, Mycoplasma, Peptostreptococcus* which are identified as biomarker in our study population, are well known bacteria associated with OC

(Bik et al. 2010; Mok et al. 2017). Our study population also reports the presence of *Pseudomonas* as a biomarker for OC which has been reported only once before (Al-hebshi et al. 2017). *Paludibacter* and *Bifidobacterium* have also been identified as a microbial biomarker in our oral cancer study population for the first time, although *Bifidobacterium* has been used to deliver anti-tumor vaccines using various novel techniques (Shirakawa and Kitagawa 2018). Conversely, the genera observed in higher abundance in control population, namely *Neisseria, Veillonella, Rothia* and *Actinobacillus* along with *Corynebacterium* were identified as biomarkers for control population. The higher abundance of these bacteria in healthy oral cavity is well known and reported. In addition, cladogram obtained confirms the presence of greater number of gram-negative genera and phyla as biomarker for oral cancer as compared to gram-positive for control and tobacco chewing population (Bolz et al. 2014).

The assessment of various diversity indices helped us understand within-group (alpha) and between-group (beta) diversity. Various alpha indices were assessed such as ACE and Chao1 to evaluate species richness, Observed OTU's for evaluation of number of OTUs, Goods coverage for evaluation of coverage of sequencing, Pielou_e to understand evenness index and Shannon and Simpson to evaluate diversity within group. All the alpha diversity indices depicted highest diversity within tobacco group, followed by control group and lowest diversity within OC group. On the other hand, beta diversity analysis was performed using PERMANOVA, PERMDISP and ANOSIM. The three indices concluded significant beta diversity between control and OC group and tobacco and OC group. PERMANOVA demonstrated significant beta diversity between control and tobacco group as well, but PERMDISP did not concur with the result, suggesting the centroids of the two groups are significantly different, but the dispersion caused due to samples makes them biologically non-significant and hence not as diverse. The PCA Jaccard, Bray Curtis, and Unifrac plots also display the same results where tobacco and control samples cluster together whereas the OC samples are away from this cluster. This suggests the diversity between control and tobacco microbiota is similar whereas that of OC group is diverse than the other two groups.

The functional prediction depicted enriched pathways related to amino acid biosynthesis and sugar fermentation in healthy controls, as has been reported earlier (Al-Hebshi et al. 2019). On the other hand, fatty acid and lipid biosynthesis pathways were enriched in diseased

samples (Liu et al. 2012). The tobacco group demonstrated enriched pathways related to reductive TCA cycle.

The abundances of bacteria in the three study groups, the biomarkers, alpha and beta diversity suggest significant diversity between control and OC group, whereas the Tobacco group lies in between control and OC group, suggesting a transition of microbiota from healthy control to OC through tobacco group.

Simultaneously, we developed a new method for absolute quantification of oral bacteria, to eliminate the drawbacks of 16S rRNA in quantitation approach. The 16S rRNA gene is widely acceptable for all microbial ecology purposes due to it conserved and variable regions, which help distinguish bacteria up to species level. One major drawback of this gene is presence of varying number of multiple copies throughout the microbial kingdom which leads to inaccuracy while enumeration (Buchan and Ledeboer 2014). The *rpoB* gene was an alternative to 16S rRNA, with similar capability to distinguish bacteria up to species level, but also being a single copy gene, aids in accurate enumeration. The housekeeping function ensures presence in every bacterial cell therefore acting as an ideal candidate (Case et al. 2007).

Increased abundance *P. gingivalis* was observed in Tobacco chewers and Oral cancer patients as compared to healthy controls (Wang et al. 2014; Ha et al. 2016). Similarly, increased levels of *F. nucleatum* was found in oral cancer patients as compared to healthy individuals. The increased abundance of *C. gingivalis, P. melaninogenica* and *V. parvula* in oral cancer patients as compared to healthy individuals was seen in present study, and has been previously reported (Mager et al. 2005; Al-hebshi et al. 2017; Zhao et al. 2017). Similarly increased abundance of *H. parainfluenzae* and *R. mucilaginosa* with healthy controls has been recorded (Chocolatewala et al. 2010; Yang et al. 2018).

Vast amount of information is published about the role and possible mechanism of action of periodontal pathogens such as *P. gingivalis* and *F. nucleatum* in causation of oral diseases, including oral cancer. The most common mechanisms of action of bacteria include chronic infection and inflammation, nitrosation of compounds and metabolism of potentially carcinogenic substances (Chocolatewala et al. 2010). *In-vitro* cell line interactions have also shown a dysregulation caused to oral cells on exposure to *P. gingivalis*. Upregulation in expression of B7–H1 and B7-DC receptors involved majorly in supressing immune system in cancer have been detected on exposure of various oral cell lines to *P.gingivalis* (Groeger et al.

2016, 2017). *Fusobacterium nucleatum* is a gram-negative adhesive oral commensal, which turns into a pathogen under certain conditions. The most common adhesin/virulence factor studied is FadA, which is also used as a diagnostic marker for the detection of *F. nucleatum* and mediates direct invasion into host cells (Fardini et al. 2011). *F. nucleatum* is also known to stimulate high secretion of IL-8, which has been characterised in cancer cells (Han et al. 2000).

For *H. parainfluenzae* and *R. mucilaginosa*, the absolute abundance of these bacteria in oral samples depicts a pattern, but no significant difference was observed. The pattern of abundance of bacteria mentioned in this study correlates well with earlier reports, thereby supporting the results obtained. The abundance of *H. parainfluenzae* was observed to be higher in control population as compared to OSCC patients, suggested by NGS data (Perera et al. 2018). On the contrary, population of *P. melaninogenica* was reported higher in OSCC patients as compared to healthy individuals (Mager et al. 2005; Zhao et al. 2017). Our results showed that the real-time PCR based assay can be used for absolute quantification of bacteria. Using *rpoB* gene as the target is better for absolute quantification, as it is a single copy gene per cell, as compared to 16s rRNA, which has variable copy number per cell. In addition, *rpoB* is a species-specific gene, thus leading to a specific amplification. By using this method, we have successfully calculated absolute abundance of eight different oral bacteria, which have been reported to show variable abundance in control population as compared to oral cancer patient microbiota.

As per literature, *S. mutans* is an important bacterium of the healthy oral cavity, yet it is associated with dental caries or in individuals using dental prosthesis. It exploits three virulence factors related with carcinogenicity once well established in dental cavities or surrounding the dental prosthesis. The first factor is synthesis glycans, ability to become more acid tolerant, and production of lactic acid (Alanazi et al. 2018). Glycans influence host cell–cell adhesion via changing the amount of functional E-cadherin at the cell–cell barrier, facilitating cancer cell separation and invasion. Various investigations have found that cancer patients have altered N-glycosylation of cell surface glycoproteins and that complex N-glycans are prevalent during tumour growth (Pinho and Reis 2015). We started out to investigate the altered gene expression in oral cell line after infection with *S. mutans*. Cytotoxicity assay depicted approx. 100% host cell viability after infection with *S. mutans* for up to 5hrs. 24hrs

infected cells depicted up to 50% cell death. Previous reports studying host-microbe interaction have reported similar cell death at longer incubation periods and therefore smaller incubation periods with MOI 100 are often chosen to study gene expression (Cho et al. 2014; Johnson et al. 2015). Further, microscopic examination demonstrated interaction of *S. mutans* with host cells only after 3hrs of infection followed by microcolony formation depicting active replication of bacterial cells. Similar to cytotoxicity assay, 24hrs infection depicted host cells undergoing apoptosis/necrosis. Further gene expression study depicted significant upregulation of Stathmin, CyclinD1 and BCl2, genes related to invasion, proliferation and survival respectively. These genes are intermediates of pathways associated with one of the hallmarks of cancer (Hanahan and Weinberg 2011). Thereby, in this preliminary study, we suggest the activation of pathways involved in cancer due to the infection with *S mutans*.

Chapter 8: Summary and Conclusion

In summary, the Indian oral microbiota was identified using 16S rRNA metagenomics approach using Illumina Mi-Seq Sequencing followed by analysis using QIIME2™ pipeline. Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria were the major phyla observed in all study subjects. A total of 16 genera contributed up to 85% of total bacterial diversity of which *Streptococcus, Neisseria, Rothia* and *Veillonella* depicted higher abundance in control population, whereas *Prevotella, Fusobacterium* and *Porphyromonas* displayed higher abundance in OC patients. Microbial biomarkers were identified for each study group using LEfSe analysis. On assessing the alpha diversity in study groups, maximum alpha diversity was observed in tobacco chewers, followed by control and least in OC patients. On the other hand, beta diversity demonstrated maximum diversity between control and OC as well as tobacco and OC study groups, whereas diversity between control and tobacco study groups was lower and these groups were similar to each other. Following diversity analysis, the functionally differential pathways expressed were predicted using PICRUSt2, which suggested Pathways related to amino acid synthesis were comparatively overexpressed in control population, those related to reductive TCA overexpressed in tobacco groups, whereas pathways for lipid biosynthesis and fatty acid elongation were overexpressed in OC population.

Further, to overcome the drawback of use of 16S rRNA in microbial ecology for enumeration purpose, *i.e.,* presence of multiple copies of 16S rRNA in every bacterial cell, we developed a simple, cost effective, sensitive and specific method using single copy *rpoB* gene for absolute enumeration of bacteria in oral samples. Using this method, we performed enumeration of eight significant bacteria involved in OC in our samples. The abundance of each bacteria in all three study groups correlated well with the reports published earlier, thereby providing confidence in the method reliability. For the enumeration of bacteria, additional characteristics such as sex, age, dietary habits, cancer stage, and cancer locations were also evaluated.

Further, to explore host-bacterial interaction *in-vitro*, we infected OC cell line with a known oral bacterium, *S. mutans*. Microscopic visualization depicted interaction/adherence between oral cells and bacteria between 1hr to 7hrs. mRNA expressions depicted significantly

increased expression of Stathmin, CyclinD1, BCl2, and Bax:Bcl2 ratio and decreased expression of FGF5 which gave an indication towards involvement of cancer related pathways.

Hence, our study helped identifying oral bacterial diversity and microbial biomarkers in OC and tobacco chewers, which can be further validated before implementing in clinical settings. Also, the role of genes identified to be upregulated in oral cell line on infection with *S. mutans* can be elucidated and confirmed by protein expression studies.

Chapter 9: Significance of the Study

Our study brings to light the composition of oral microbiota of healthy, long-term tobacco chewers and OC patients in the Indian population. With the ever-increasing cases of OC and related mortality in India and the unveiling of role of microbiota in oral cancer, our study would help in understanding the diversity in the oral microbiota and aid in comparing it with the global reports. The microbial biomarkers identified along with the qPCR method developed would also aid in early diagnosis and prediction of individuals at high risk of developing oral cancer, especially long-term tobacco chewers. At the same time, the preliminary assessment of bacterial infection in the oral cell line opens new doors to explore the mechanism by which bacteria can promote tumorigenesis.

Publications and Presentations

Publications

1. **Sawant S**, Dugad J, Parikh D, Srinivasan S**, Singh H**. (2021) Identification & correlation of bacterial diversity in oral cancer and long- term tobacco chewers- A case- control pilot study. *Journal of Medical Microbiology*. 70(9), 001417. (**IF:2.35)**
2. **Sawant S,** Dugad J, Parikh D, **Singh H.** (2021) Absolute quantitation of oral bacteria involved in oral cancer by real-time PCR. *Medicine in Microecology*. 7, 100034.
3. Kamble A, **Sawant S**, **Singh H**. (2020) 16S ribosomal RNA gene-based metagenomics: A review. *Biomedical Research Journal* 7.1: 5.

Conferences and poster presentations

1. The **60th Annual Conference of Association of Microbiologists of India (AMI-2019)** and International Symposium on "Microbial Technologies in Sustainable Development of Energy, Environment, Agriculture and Health", Haryana, India (15th-18th November'19): presented poster entitled "Absolute quantitation of oral bacteria involved in oral cancer by real-time PCR"
2. **18th Asia Pacific Congress of Clinical Microbiology and Infection, Singapore (11th-13th November'21)** presented poster entitled "Identifying oral microbiome diversity of Indian tobacco chewing population and patients with oral cancer using next generation sequencing."
3. Attended "Advances in Materials Science & Applied Biology" 2019, NMIMS, Mumbai.

Scholarship

1. Women's Graduate Union Scholarship 2020-21

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